International Immunopharmacology 72 (2019) 330–338

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Geniposide, a sonic hedgehog signaling inhibitor, inhibits the activation of hepatic stellate cell

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ARTICLE INFO

Keywords:
Geniposide
Liver fibrosis
Sonic hedgehog signaling
Cell cycle

ABSTRACT

Liver fibrosis is a continuous wound-healing process, which is due to excessive deposition of extracellular matrix (ECM) caused by activated Hepatic Stellate Cells (HSCs). Geniposide (GP) is a naturally occurring iridoid glucoside and is extracted from *Gardenia jasminoides* Ellis. GP has long been speculated to play a vital role in the treatment of hyperlipidemia and fatty liver. Emerging evidence has demonstrated that GP may be importantly associated with the pathophysiology of liver fibrosis. Nevertheless, the fundamental mechanism is yet uncertain. This studywas designed to explore the possible mechanism for the inhibitory effect of GP on CCl_4-induced mice liver fibrosis. In line with several clinical reports, GP significantly reduced the levels of hyaluronic acid (HA), laminin (LN), hydroxyproline (HYP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Meanwhile, liver fibrosis was significantly alleviated by GP as indicated by decreased α-smooth muscle actin (α-SMA) expression and type I collagen alpha-1 (Col I α1) deposition. In addition, GP could reduce the cell viability (IC50 = 77.11 and 42.88 μM at 24 and 48 h respectively) and cause G2/M cell arrest of the activated HSC-T6 cells. Noteworthy, GP prominently suppressed the Sonic hedgehog (Shh) signaling pathway and it might inhibit the activation and proliferation of HSC-T6 cells through Shh signaling pathway. Taken together, the current research indicate that GP has a promising antifibrotic effect which could be partially attributed to its suppression of Shh signaling pathway. Hence GP could be employed as a promising therapeutic strategy for the treatment of clinical hepatic injury.

1. Introduction

Liver fibrosis is supposed to be a wound-healing reaction of liver damage, due to various etiologies of chronic liver damage, such as alcohol, viral infection, toxic damage and so on [1,2]. Without effective therapeutic measures, the liver will bloom into hepatic cirrhosis and eventually leads to hepatocellular carcinoma. HSCs, the primary source of ECM, are well known for its key role in liver fibrosis [3–5]. The activation of HSCs is regulated by various inflammatory cytokines and growth factors, such as transforming growth factor beta 1 (TGF-β1), platelet-derived growth factor (PDGF) and so on [6,7]. PDGF, a major profibrogenic mediator, can not only promote the activation of HSCs but also contribute to the proliferation of HSCs [8]. Thus, PDGF is used to activate HSCs in this study. On account of the activated HSCs are a vital cellular event in the process of liver fibrosis [9,10]. Therefore, clearance of activated HSCs may be helpful to reduce or even reverse liver fibrosis.

A growing body of evidence suggests that Hedgehog signaling(Hhs) is implicated in a wide range of biologic actions, including embryogenesis, tissue homeostasis, cell differentiation, cell proliferation and so on [11]. Hhs has three family ligands, including Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). Patched (PTCH1 and PTCH2), a cell surface receptor of Hhs, can interact with Hhs. Without Hhs ligands, Patched suppress Smoothened (Smo) and the Hhs is silenced. Otherwise, Smo will be liberated, which leads to a cascade that finally activates Glioblastoma (Gli) family to control its target genes, and the Hhs is activated [12,13]. Intriguingly, Shh signaling can modulate wound healing responses in adult liver tissues and

Abbreviations: GP, geniposide; ECM, extracellular matrix; HSCs, hepatic stellate cells; HA, hyaluronic acid; LN, laminin; HYP, hydroxyproline; ALT, alanine aminotransferase; AST, aspartate aminotransferase; α-SMA, α-smooth muscle actin; Col I α1, type I collagen alpha-1; PDGF, platelet-derived growth factor; Shh, sonic hedgehog; Gli1, GLI family zincfinger 1; Smo, smoothened; Hhs, hedgehog signaling

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https://doi.org/10.1016/j.intimp.2019.04.016
Received 12 November 2018; Received in revised form 6 April 2019; Accepted 7 April 2019
Available online 18 April 2019

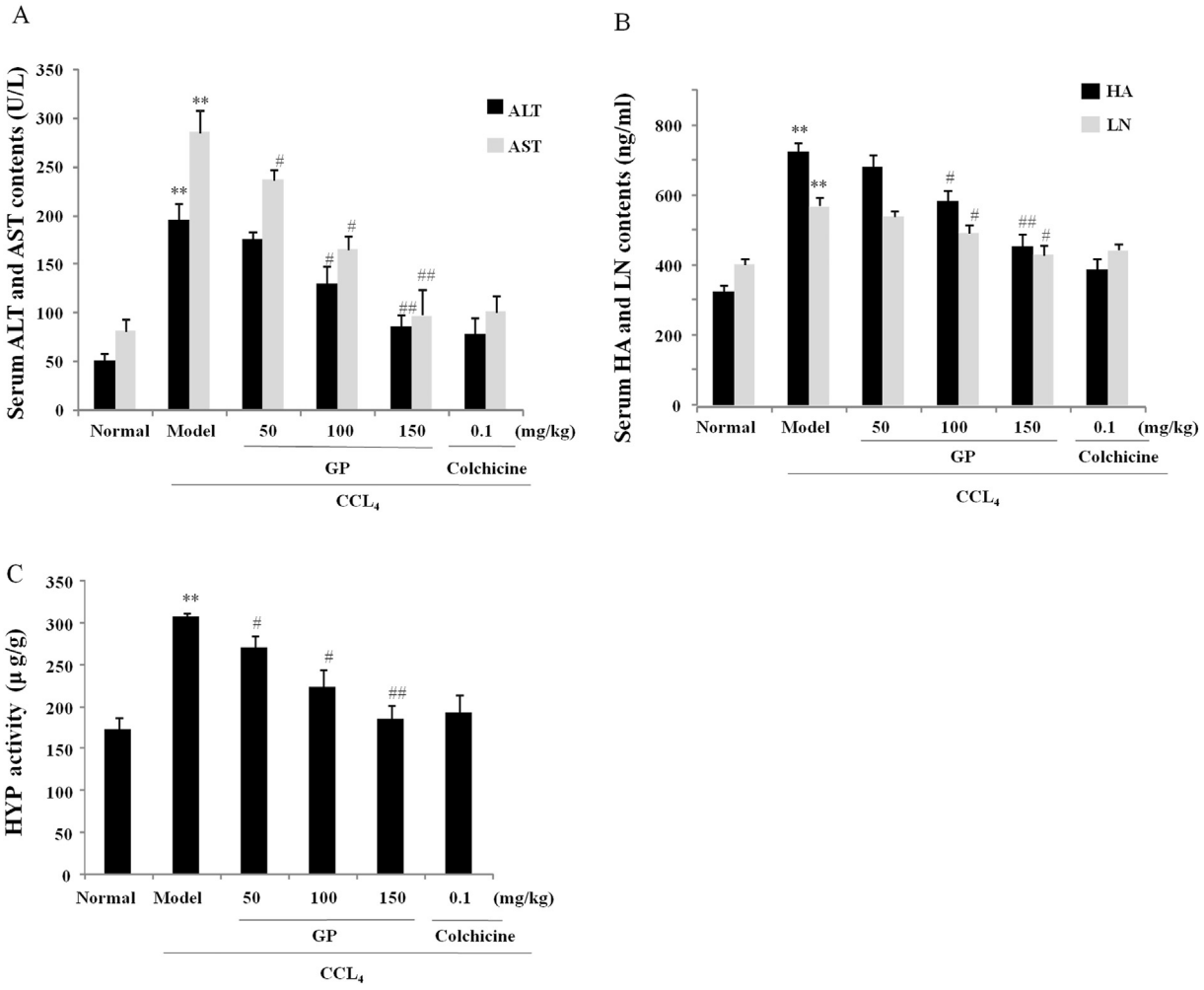


Fig. 1. GP reduced CCl_4-induced mouse liver damage. A: GP decreased serum levels of ALT and AST. B: GP decreased the levels of HA and LN(markers of liver fibrosis). C: GP reduced the expression of HYP. Data presented are the mean ± SEM, each group consists of 8 mice. Normal = mouse treatment with normal saline; Model = mouse treatment with normal saline + CCl_4; $^{**}P < 0.01$ versus Normal; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Model.

the promoting role of Shh signaling in liver fibrogenesis is correlated positively with the activation of HSCs, indicating that Shh signaling may be a potential therapeutic target for liver fibrosis [14,15].

Previous studies have proved that traditional Chinese herbal medicines can slow down the development progression of liver fibrosis [16,17]. GP is a naturally occurring iridoid glucoside obtained from *Gardenia jasminoides* Ellis, which have been used for liver disorders therapy previously [18]. In recent years, many studies are paid close attention to the potential effect of GP in liver injury and found that GP alleviated liver injury [19–21]. Despite the excellent hepatoprotective effects of GP have been proved, the underlying mechanisms remains unclear until now. In the present study, we aims to understand the possible protection of GP against CCl_4-induced mice liver fibrosis and its mechanisms underlying.

2. Materials and methods

2.1. Materials

GP (HPLC ≥ 98%), was provided by Shanghai Jinsui Biotechnology (Shanghai, China). Rat HSC-T6 cell line was purchased from Shanghai Fumeng Gene Biological Corporation (Shanghai, China). PDGF was purchased from PEPROTECH (UK). ALT, AST, HA, LN and HYP activity assay kits were obtained from Jiancheng Bioengineering Institute (Nanjing, China). Rabbit anti-Shh polyclonal antibody (bs-1544R) was bought from Bioss Biotechnology (Beijing,China). Rabbit anti-Gli1 polyclonal antibody (bs-16248R) was obtained from Bioss Biotechnology (Beijing, China). Mouse anti-β-actin monoclonal antibody was bought from Bioworld Technology (Atlanta, Georgia, USA). Secondary goat anti-rabbit and goat anti-mouse immunoglobulin (IgG) horse radish peroxidase (HRP) antibodies were bought from Santa Cruz Biotechnology (California, USA).

2.2. Animals and treatments

Male Kunming mice (18–20 g) were provided by the Experimental Animal Center of Anhui Medical University. The mice were received human care and the animal experimental processes were in accordance with the Animal Experiments Guidelines and Animal Care of Chinese Academy of Sciences. The mice were classified into six groups randomly, including normal group, model group, GP (50 mg/kg) group, GP (100 mg/kg) group, GP (150 mg/kg) group and colchicine (0.1 mg/kg) group, each group contains 25 mice. At the beginning of the experiment, the normal group was subjected subcutaneous injections of normal saline, while the other five groups were injected with CCl_4 (10% CCl_4, diluted in olive oil, 0.02 ml/g/mice) for twice a week up to 8 weeks. Then, the normal mice were subjected subcutaneous injections of normal saline and gavaged with normal saline and the model group was treated with CCl_4 and gavaged with normal saline. Meanwhile, GP groups were subjected subcutaneous injections of CCl_4 solution and

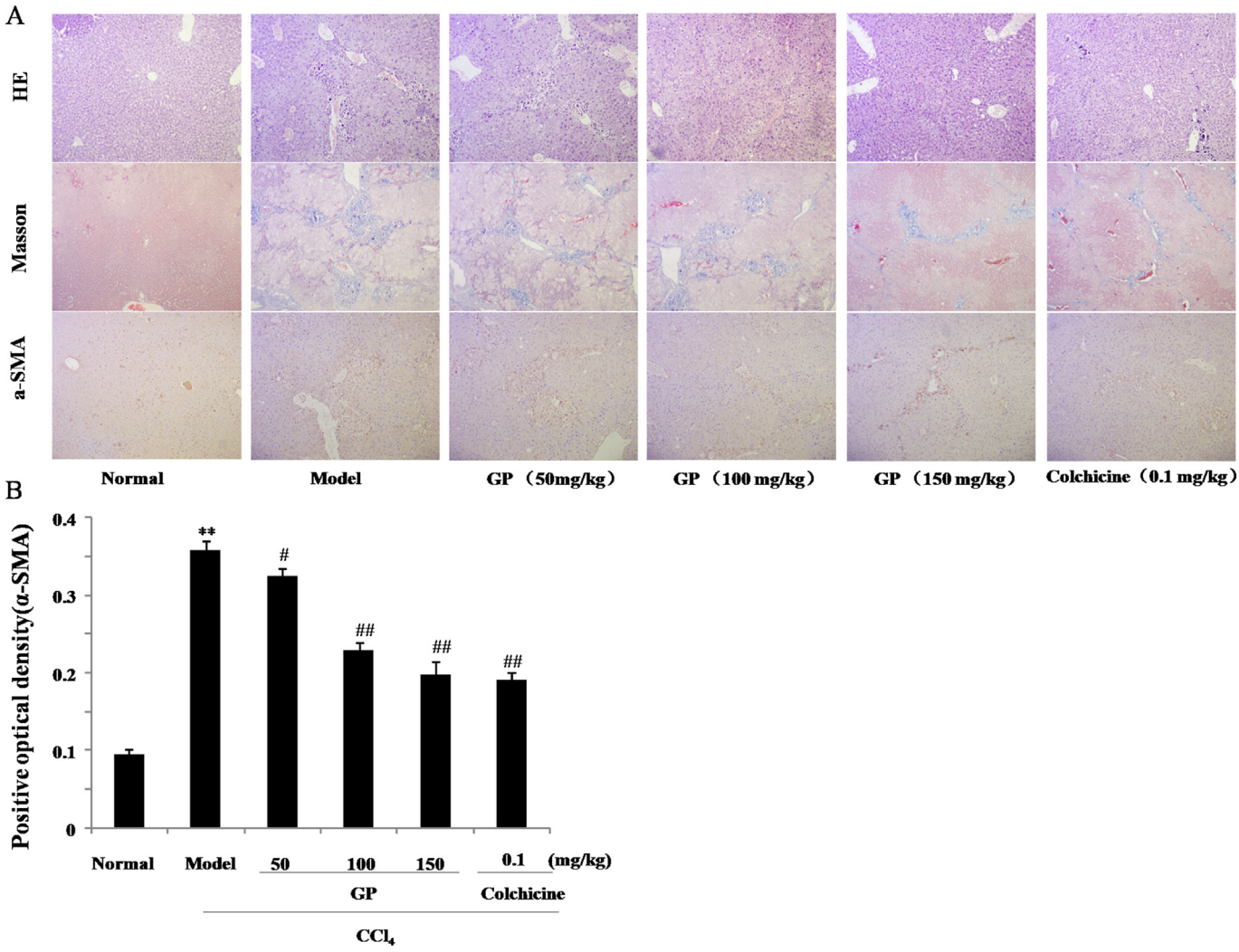


Fig. 2. GP attenuated CCl_4-induced histopathological deterioration in mice liver fibrosis. A: Hepatic tissues from different groups were detected by hematoxylin and eosin (H&E) staining (×200) and Masson staining (×200), respectively. B: The Positive optical density of a-SMA was analyzed by immuno-histochemistry. GP (100, 150 mg/kg) and colchicine (0.1 mg/kg) prominently decreased the level of α-SMA. Data presented are the mean ± SEM, each group consists of 8 mice. Normal = mouse treatment with normal saline; Model = mouse treatment with normal saline + CCl_4; $^{**}P < 0.01$ versus Normal; $^{\#\#}P < 0.01$ versus Model.

gavaged with GP (50, 100, 150 mg/kg), dissolved in 5% sodium carboxymethylcellulose and colchicine group was injected by CCl_4 subcutaneously and gavaged with colchicine (0.1 mg/kg), dissolved in a DMSO/saline solution. The addition of GP and colchicine were performed 24 h after CCl_4 treated. Six weeks latter, all mice were sacrificed under anesthesia and the liver tissues and venous blood were collected for further research. All animal experiments were supported by the Animal Experimental Committee and the Ethic Committee of Anhui Medical University.

2.3. Cell culture

HSC-T6 cells were maintained in DMEM supplemented with 10% FBS, 100 mg/ml streptomycin and 100 U/ml penicillin at 37 °C with humidified atmosphere (5% CO_2). GP was dissolved in Anddimethyl sulfoxide (DMSO) to obtain the stock solution (200 μM). The final concentration of DMSO was not exceeded 0.1% (v/v).

2.4. Cell viability assay

The 3-(4,5–dimet-hylthiazol-2-yl)-2,4-diphenyl-tetrazolium bromide (MTT) assay was conducted to determine the cell viability. HSC-T6 cells were put into a 96-well plates and treated with GP at concentrations of 0, 12.5, 25, 50, 100, 150, 200 and 400 μM. After incubation, 20 μl of MTT solution was added into each well. Four hours later, the medium was replaced with DMSO (150 μl). The optical density (OD) value at 492 nm of each well was detected by a Bio-Rad 680 microplate reader (USA).

2.5. Cell cycle analysis

Cell Cycle Analysis Kit was used according to manufacturer's instruction. 24 h later, cold PBS was used to wash the cells three times. Then, 70% ethanol was mounted in PBS at −20 °C for 24 h. After the cells were fixed, they were marked with inpropidium iodide (PI) staining buffer (0.5 mL) at 37 °C for 40 min behind the scenes. BD LSR flow cytometer was used to perform the analysis. Experiments were performed in triplicate and repeated at least three times.

2.6. RNA extraction and RT-qPCR

Total RNA was extracted from HSC-T6 cells by using TRIzol reagents (Biomic, USA) according to the manufacturer's instructions. One-step quantitative real-time PCR analyses for mRNA of Shh, GLI1 and β-actin

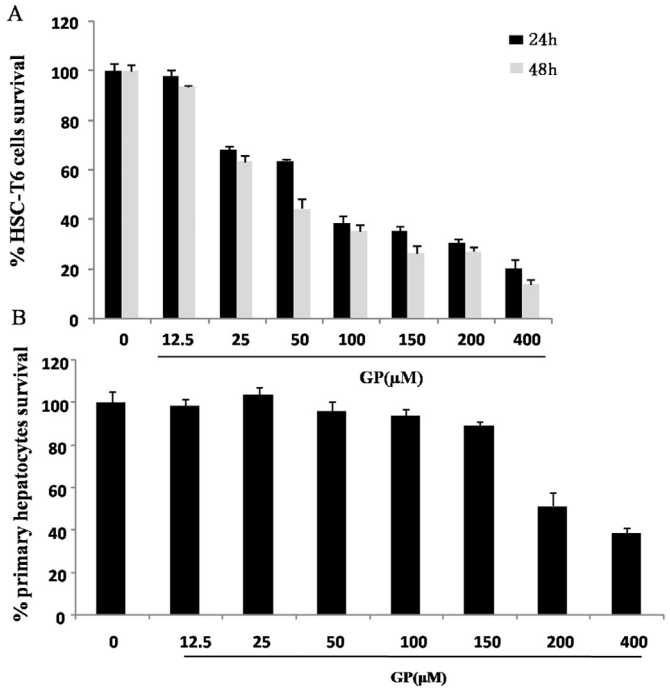


Fig. 3. GP decreased the cell viability of activated HSC-T6 cells with little effect on primary hepatocytes. HSC-T6 cells and primary hepatocyte cells were cultured and treated with GP (0, 12.5, 25, 50, 100, 150, 200, 400 μM) A: Effect of GP on the cell viability of HSC-T6 cells at 24 h and 48 h. B: The cytotoxicity of GP on primary hepatocytes at 48 h. Data presented are the mean ± SEM ($n = 3$). Data are representative of at least three separate experiments. * $P < 0.05$, ** $P < 0.01$ versus Normal; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Model.

were performed by using Thermoscript RT-qPCR kits (Fermentas, USA) in an ABI Prizm step-one plus real-time PCR System (Applied Biosystems, USA). The mRNA of β-actin was used as an internal control. The fold-change for target genes relative to β-actin was determined by the formula $2^{-\Delta\Delta Ct}$. All experiments were performed in triplicate and repeated at least three times.

2.7. Western blot analysis

HST-T6 cells and mice liver tissues were dissolved by RIPA lysis buffer (Beyotime, China), respectively. Total protein (30 or 50 mg) from cells or tissues were separated by SDS-PAGE and then transferred to a PVDF membrane. After that, the PVDF membrane was incubated for 3 h. Then, the PVDF membrane was put into primary antibodies solution and incubated at 4 °C all night long. Mouse anti-α-SMA and anti-Col I α1 were diluted 1:500 with PBS. Rabbit polyclonal anti-Gli1 and anti-Shh were diluted 1:300. On next morning, PVDF membrane was washed four times in every 10 min. Then, PVDF membrane was incubated in anti-mouse or anti-rabbit antibodies solutions for 1 h at 37 °C. Finally, proteins were detected by ECL-chemiluminescent kit.

2.8. Statistical analysis

Data are presented as mean ± SD. The data were analyzed by SPSS software. Hypothesis testing methods contained Student's *t*-test comparison and one-way analysis of variance. Differences were considered to be statistically significant (* $P < 0.05$, ** $P < 0.01$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\$}P < 0.05$).

3. Results

3.1. Effect of GP on liver damage

Serum levels of ALT and AST, reflect the degree of liver injury, were detected respectively. Compared to the normal group, ALT and AST serum levels in model group were prominently elevated. However, they were markedly decreased in GP (100, 150 mg/kg) groups (Fig. 1A). In addition, HA and LN activities can reflect the degree of liver fibrosis. The result showed that the levels of HA and LN were significantly increased in model group whereas the addition of GP reduced their levels (Fig. 1B). Furthermore, compared to model group, GP significantly reduced the level of HYP (Fig. 1C). These data indicated that GP could prevent the mice from liver damage.

3.2. Effect of GP on mice histopathological deterioration

HE staining showed that the CCl_4-treated group had severe inflammatory cells infiltration, steatosis and numerous fibrous septa. However, these pathological changes were relieved by GP treatment. In addition, Masson staining showed the model group liver section exhibited excessively deposited collagen fibers around the central vein and portal triad. Nevertheless, collagen fibers were markedly reduced in GP treatment groups (Fig. 2A). Moreover, the immunostaining signal of α-SMA was increased in model group while decreased in GP groups (Fig. 2B). These results suggested that GP could amend CCl_4-induced mice liver histopathological deterioration.

3.3. Effect of GP on cell viability

We initially explored the administration of GP on the viability of cultured HSC-T6 cells by using MTT assay. As shown in Fig. 3A, the addition of GP significantly inhibited the growth of HSC-T6 cells in a dose- and time-dependent manner. The 50% inhibition concentration (IC 50) of GP on HSC-T6 cells was 77.11 and 42.88 μM at 24 and 48 h respectively. However, a useful antifibrotic drug must be selective for HSC cells. Thus, we examined the cytotoxicity of GP on primary hepatocytes. Fortunately, GP (0-150 μM) caused little effect on the primary hepatocytes. Accordingly, we chose the concentrations of GP (25, 50, 100 μM) in subsequent assays. Taken together, GP could inhibit the viability of activated HSC-T6 cells.

3.4. Effect of GP on α-SMA and Col I a1 expression

The protein levels of α-SMA and Col I a1 were evaluated both in vitro (Fig. 4A) and in vivo (Fig. 4B). In activated cells, the increasing α-SMA and Col I a1 were reversed by GP apparently. Consistently, compared to CCl_4-treated group, the levels of α-SMA and Col I a1 were significantly decreased in GP groups.

3.5. Effect of GP on the cell cycle of activated HSC-T6

In order to explore the potential mechanism for the inhibitor effect of GP on activated HSC-T6 cells, flow cytometry analysis was performed. As shown in Fig. 5, it was clearly evident that GP caused a G2/M arrest. This observation demonstrated that GP could suppress the proliferation of activated HSC-T6 cells. However, further studies are still needed to clarify this standpoint.

3.6. Effect of GP on Shh signaling pathway

In order to illuminate the molecular mechanism for the anti-fibrotic effect of GP, the protein levels of Shh and Gli1 were evaluated in CCl_4–induced mice liver fibrosis model. They were increased in CCl_4–treated group and decreased in GP administration (Fig. 6A). In addition, we also measured the miRNA and protein levels of Shh and

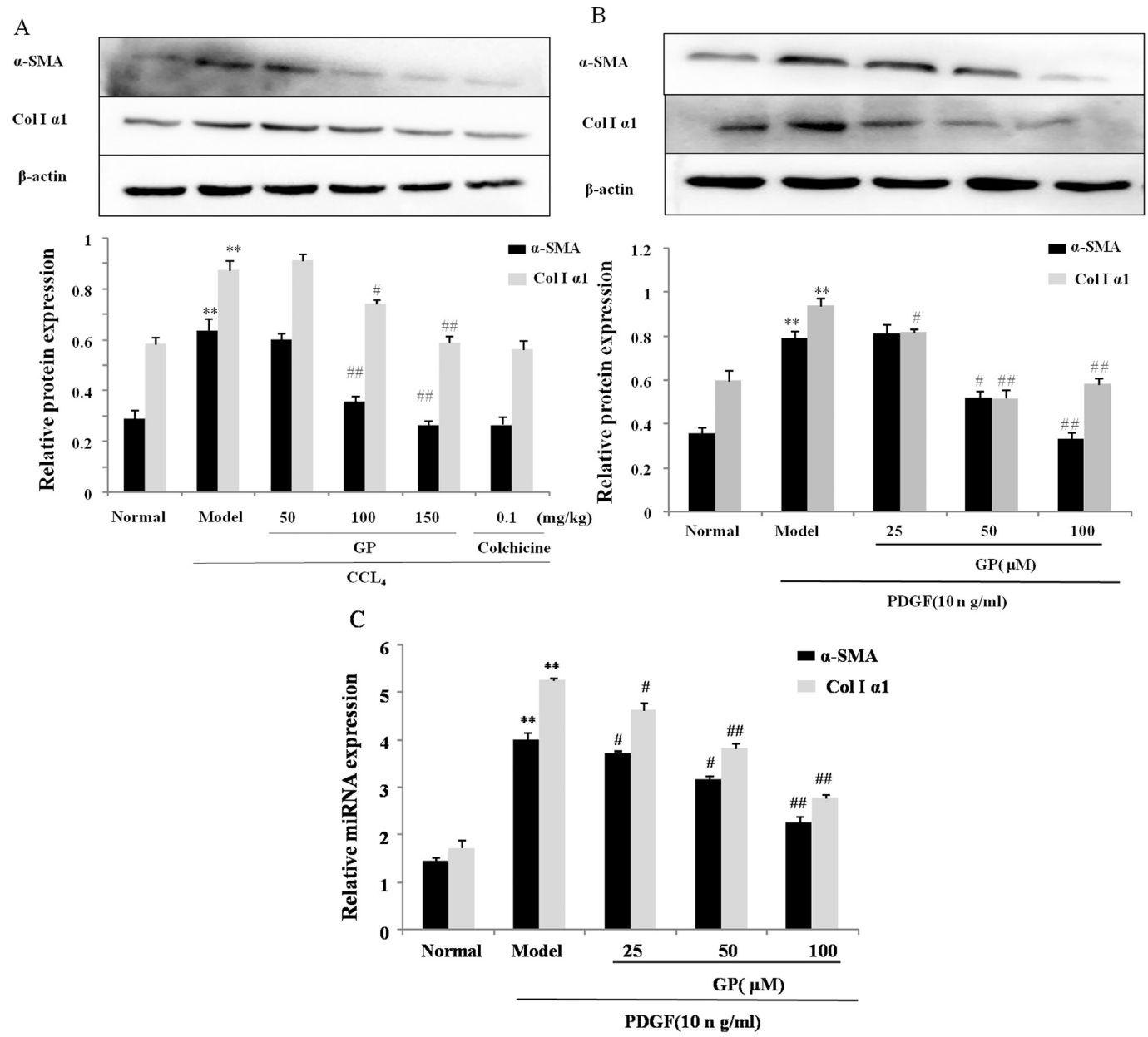


Fig. 4. GP decreased the levels of α-SMA and Col I α1. Western blot analysis of protein expression levels in activated HSCs and in mice liver fibrosis tissues (A and B).The enhanced protein level of α-SMA and Col I α1 in model group were decreased by the addition of GP. C: GP decreased the mRNA level of α-SMA and Col I α1 in activated HSC-T6 cells. Data presented are the mean ± SEM (n = 3). Data are representative of at least three independent experiments. **P < 0.01 versus Normal; #P < 0.05, ##P < 0.01 versus Model.

Gli1 in activated HSC-T6 cells. The expression of Shh and Gli1 were decreased in GP treated cells compare to PDGF-BB treated cells (Fig. 6B, C). Moreover, we also explored the role of GP on Shh signal in primary hepatocytes. As shown in Fig. 6D and E, GP (25, 50, 100 μM) did not inhibit the expression of Shh and Gli1, indicating that GP had selective inhibitory effects on Shh signal in HSCs. These results suggested that GP could be a negative regulator of Shh signaling pathway in activated HSCs.

3.7. GP inhibited the activation and proliferation of HSC-T6 cells through suppression of Shh signaling

To further determine whether the protective effect of GP on liver fibrosis was connected with its inhibitor role on Shh signaling pathway, cyclopamine (10 μM) was used to block Shh signaling pathway. As shown in Fig. 7, compared with model group, cyclopamine (10 μM) treated group significantly reduced the expression of Shh and Gli1. Higher amount of α-SMA and Col I a1 were expression in model group compared with normal group. However, they were remarkably reduced in GP (100 μM) treatment group. It was noteworthy that the expression of α-SMA and Col I a1 were decreased more in GP (100 μM) group compared with GP (100 μM) and cyclopamine (10 μM) co-treatment group. Furthermore, our study also found that the inhibitor role of GP on the cell cycle and cell viability of HSC-T6 cells was significantly blocked down by the addition of cyclopamine (Fig. 7B, C). Taken together, the present results indicated that GP could improve the hepatic fibrosis partly via targeting Shh signaling pathway.

4. Discussion

Liver fibrosis, a wound-healing response of liver, is characterized by gradually deposition of ECM [22]. Accumulated evidence showed that

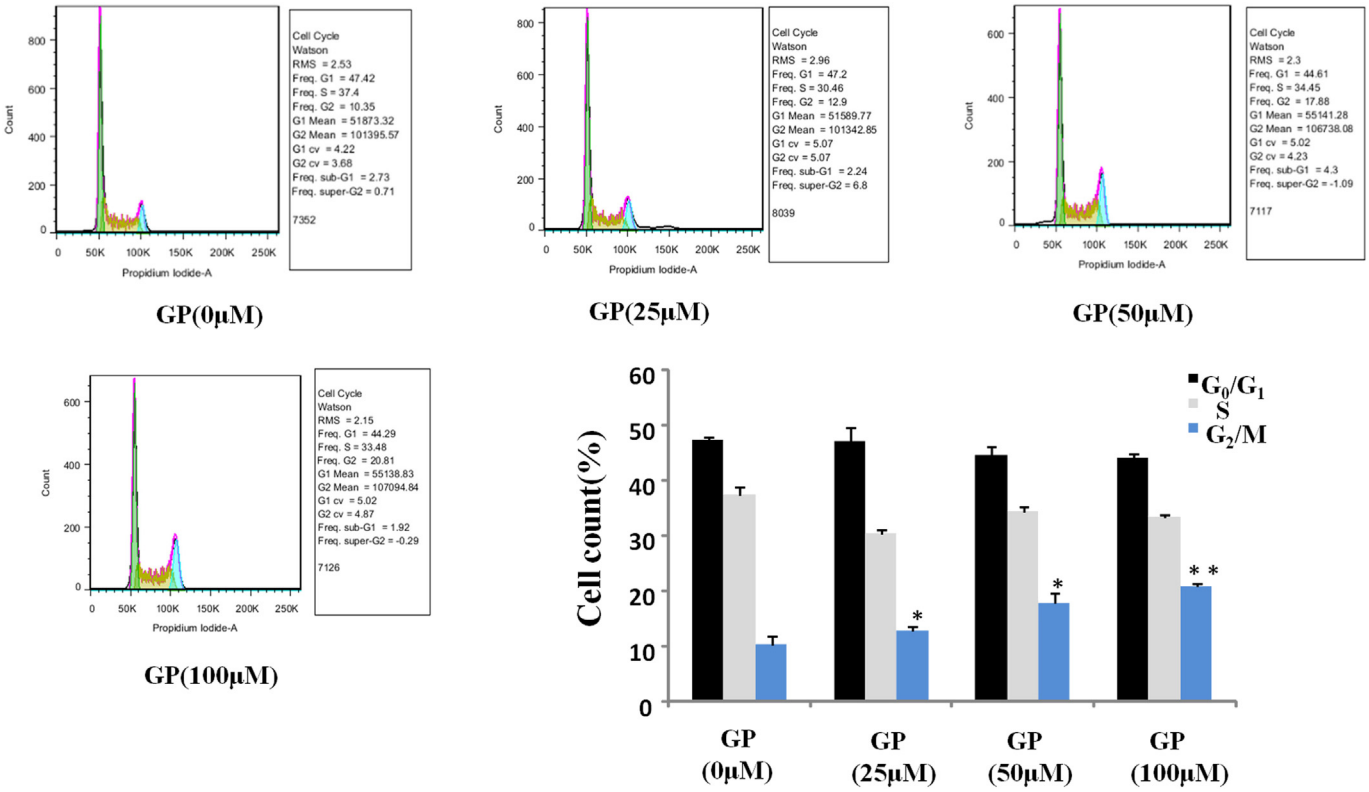


Fig. 5. GP caused G2/M arrest of activated HSC-T6 cells. The inhibitory role of GP on cell cycle of activated HSC-T6 cells was analyzed by Flow cytometry. Data presented are the mean ± SEM (n = 3). Data are representative of at least three separate experiments. * P < 0.05, **P < 0.01 versus Model.

reducing the inflammatory cytokines released by ECM might improve or even reverse liver fibrosis [23]. Activated HSCs, the primary source of ECM, is provide key contributions to development progress of hepatic fibrosis. And inhibiting the activation and proliferation of HSCs was deemed be a feasible approach for the treatment of liver fibrosis [24–26]. Therefore, finding of novel drugs for reversing or halting activation and proliferation of HSCs is an urgent demand.

GP is a naturally occurring iridoid glucoside extracted from *Gardenia jasminoides* Ellis. Previous studies have demonstrated the antifibrotic effect of GP [27]. However, the underlying mechanism remains incompletely understood. In the current research, GP significantly reduced some fibrotic index, including ALT, AST, LA, HA and HYP, which in consistent with the data obtained from the group of Tao-tao Ma in mice liver injury tissues [28]. In addition, HE staining, Masson staining and immuno-histochemistry also proved GP could amend CCl_4-induced mice liver histopathological deterioration. In vitro and in vivo experiments, activated HSCs were conspicuously inhibited by GP as indicated by reduced α-SMA and Col I a1. Furthermore, our result demonstrated that GP significant reduced cell viability and caused a G2/M cell cycle arrest in activated HSC-T6 cells.

Hgs is a highly conserved signaling pathway, which was originally identified from Drosophila [29]. There has been evidence showing Hgs eliciting a wide variety of biological responses, such as cell proliferation, adhesion, migration, and so on [30,31]. The interaction between the ligand (Shh) and cell surface receptor (ptch) is a crucial step in the activation of Shh signaling. This interaction releases Smo of which is inhibited by ptch, result in the activation of Gli transcription factors. Upon activation, the downstream target genes of Gli will be activated, including those regulating cell viability, cell cycle, cell apoptosis and so on [32,33]. Emerging evidence has indicated the Hgs is implicated in the activation of HSCs [34]. This suggests that Shh may be a novel therapeutic target for liver fibrosis. In this study, GP significantly reduced the levels of Shh and Gli1 in activated HSC-T6 cells and in mice model of liver fibrosis. In addition, we investigated whether GP inhibit Shh in normal hepatocytes. The result demonstrated that GP did not reduce the level of Shh and Gli1 in primary hepatocytes, indicating that the inhibitor role of GP on activated Shh signaling in HSCs.

To examine the underlying mechanism of GP on the activation and proliferation of HSC, cyclopamine (10 μM) was used to block the Shh signaling. We found that GP significantly reduced the expression of α-SMA and Col I a1. However, these effects were blocked down by the suppressed Shh signaling, implying that GP inhibits the activation HSC-T6 cells, at least in part, via Shh signaling pathway. Noteworthily, there has evidence showing that knocking down of Gli1 could facilitate G2/M cell cycle arrest in human chondrosarcoma cells [35]. In addition, recently research demonstrated that activated Shh signaling could facilitate G2/M transition and promote cell proliferation in human hepatocellular carcinoma cell lines [36]. Thus, we hypothesis whether the G2/M cell cycle arrest caused by GP is relevant to its inhibition of Shh signaling. The result showed that the cell cycle arrest role of GP was reduced by the blocked Shh signaling. Furthermore, MTT result demonstrated the inhibitor role of GP on cell viability was reversed by the silencing of Shh signaling.

To sum up, our present study suggested that GP ameliorated mice liver fibrosis, which might function partly via Shh signaling pathway. Given these results, the regulatory role of GP on Shh signaling pathway may establish a novel therapeutic strategy for the treatment of hepatic fibrosis. Nevertheless, further studies are still needed to verify this opinion.

Acknowledgements

This project was supported by the National Natural Science Foundation of China (81473268).

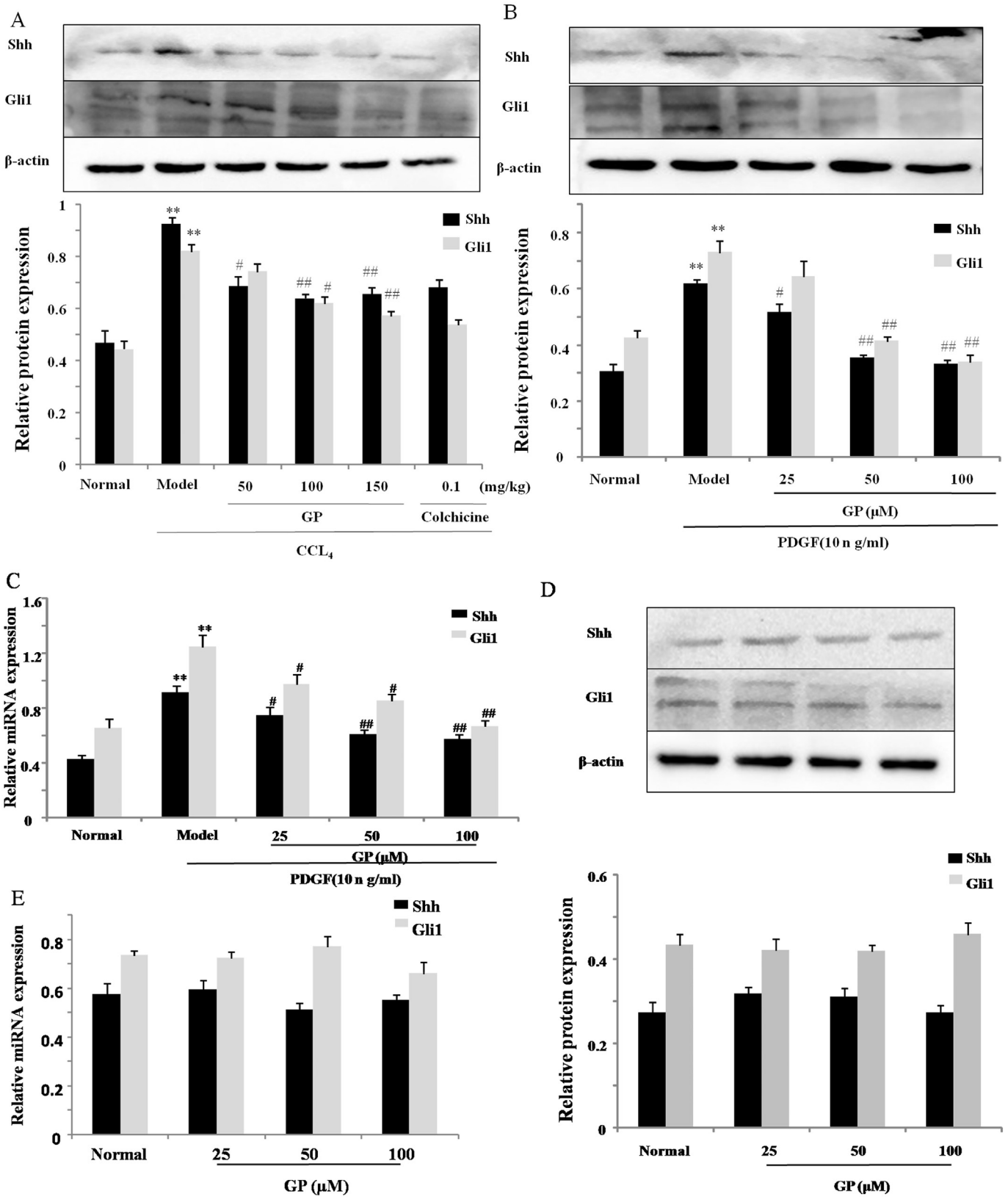
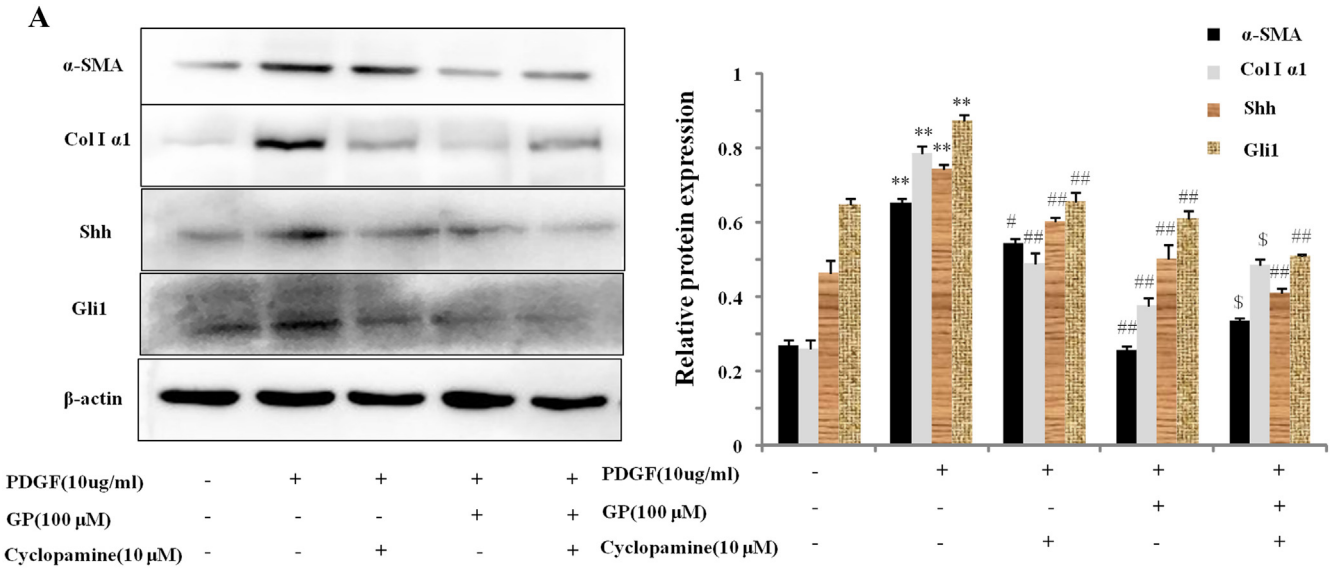
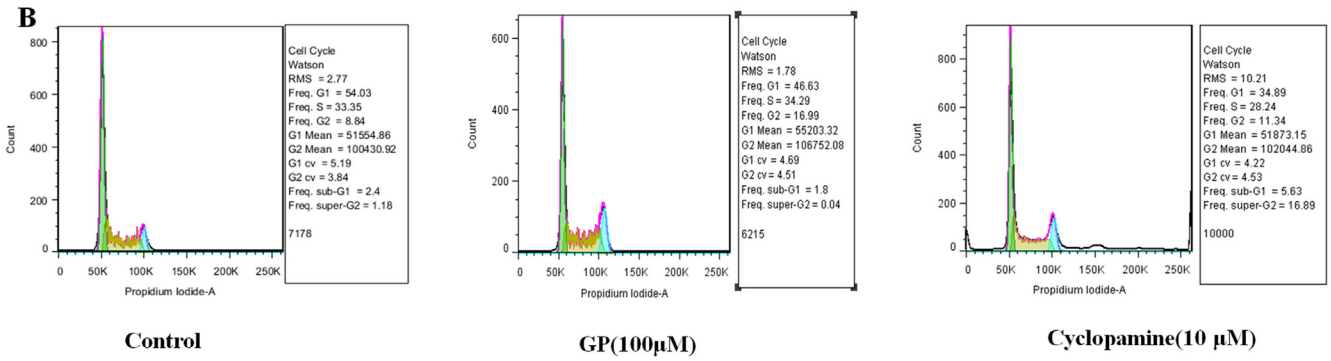
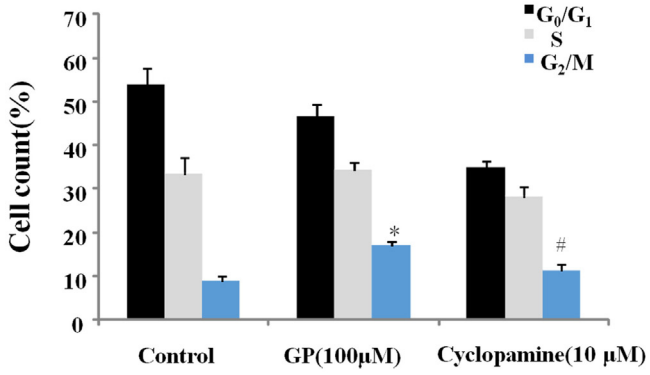


Fig. 6. GP inhibited the Shh signaling in activated HSC-T6 cells. A: GP decreased the protein levels of Shh and Gli1 in liver fibrosis tissues. B and C: GP decreased the miRNA and protein levels of Shh and Gli1 in activated HSC-T6 cells. D: GP (25, 50, 100 μM) did not reduce the expression of Shh and Gli1 in primary hepatocytes. The expression of β-actin was used as an internal control. Data presented are the mean ± SEM(n = 3). **P < 0.01 versus Normal; #P < 0.05, ##P < 0.01 versus Model.







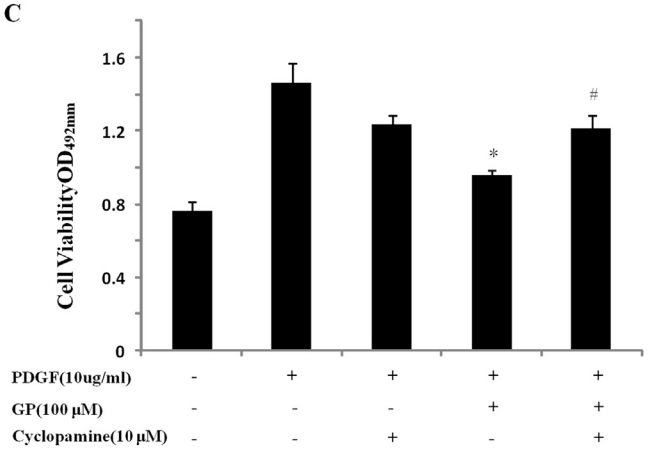


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Fig. 7. The anti-fibrotic role of GP may be via Shh signaling pathway. Cyclopamine (10 μM) was used to block the Shh signaling pathway. A: The expression levels of α-SMA, Col I α1, Shh and Gli1 protein were determined by western blotting. The inhibitor role of GP on cell viability (B) and cell cycle (C) was reversed by blocked Shh signaling pathway. Data presented are the mean ± SEM (n = 3). Data are representative of at least three separate experiments. *P < 0.05, **P < 0.01 versus Normal; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 versus PDGF (10μg/ml); $^{\$}$P < 0.05 versus PDGF (10μg/ml) + GP (100 μM).

Conflict of interest

We declare that we have no conflict of interest.

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